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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	H. Zaghouani	)	Group Art Unit 1664
Appl. No.	:	08/779,767	)	
Filed	:	January 7, 1997	)	
For	:	COMPOUNDS, CONDITIONS, AND METHODS FOR THE ENDOCYTIC PRESENTATION OF IMMUNOSUPPRESSIVE FACTORS	)	
Examiner	:	P. Nolan	)	

I hereby certify that this correspondence and all marked attachments are being deposited with the United States Postal Service as first-class mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231, on

September 20, 2001
(Date)

Daniel Hart
Daniel Hart, Reg. No. 40,637

DECLARATION UNDER 37 CFR 1.131

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

I, Habib Zaghouani, declare as follows:

1. I am the inventor of claims 4, 6, 9, 11-21, 24-27, 29-70, and 72-75 of the application identified above and inventor of the subject matter described and claimed therein.

2. Prior to November, 1996, I had completed my invention as described and claimed in the subject application in this country, as evidenced by the following:

a. Prior to November, 1996, I conceived the idea of using an immunoglobulin or a portion thereof, which is capable of binding to an Fc receptor, linked to a T cell receptor antagonist peptide for the endocytic presentation of antagonists on the surface of antigen presenting cells in association with MHC Class II molecules thereby preventing T cell activation.

b. Prior to November, 1996, I communicated my conception of the invention to a graduate student, Kevin Legge, who was working under my supervision and direction in my laboratory at the University of Tennessee. I instructed Kevin Legge to perform a series of experiments, that I conceived, that were designed to reduce the invention to practice. Kevin Legge performed those experiments in

accordance with my instructions and under my direction. I worked with Kevin Legge to perform those experiments. Those experiments are summarized in a laboratory notebook kept by Kevin Legge, and 23 pages of that notebook are attached hereto as Exhibit A. Note that the dates have been deleted from Exhibit A.

Page 1 of Exhibit A presents the sequence of the oligonucleotide primers designed for the creation of the chimeric antibody containing the PLP-LR sequence (which encodes a T cell receptor antagonist) by PCR mutagenesis. These primers were designed to add the PLP-LR coding sequence into the variable region of the heavy chain fragment. The primers were custom synthesized by Gibco/BRL, as indicated on the labels included on page 1. The certificate of analysis prepared by Gibco/BRL is shown on page 2.

Page 3 of Exhibit A describes the PCR used to create the PLP-LR fragment. A plasmid containing an immunoglobulin heavy chain fragment was used for a template. Two separate PCR reactions were performed using the primers from page 1. In one reaction, a fragment of the immunoglobulin heavy chain variable region containing an *Nco*1 site on one end with the antagonist peptide-encoding sequence (PLP-LR) at the other end was prepared. To do this, PCR was performed with the plasmid containing the immunoglobulin gene as the template, combined with an oligonucleotide primer that confers an *Nco*I site to the fragment (sequence not shown), and the above mentioned PLP-LR primer (designated Nco-PLP-LR) which will add the nucleic acid encoding the PLP-LR sequence to the other end of the fragment. An *Eag*I site was engineered into the PLP-LR sequence (as shown on page 1).

The other reaction was designed to create a fragment of the immunoglobulin heavy chain variable region with a newly engineered *Apa*I site at one end, and the antagonist peptide (PLP-LR) containing an engineered *Eag*I site at the other end. The same template (the plasmid containing the immunoglobulin gene) was used for this reaction, along with the following two primers: one primer conferring an *Apa*I site to the fragment (sequence not shown), and another primer conferring the sequence encoding the PLP-LR peptide sequence (designated APA-PLP-LR). Agarose gel electrophoresis of the PCR fragments revealed that the *Nco*I reaction produced the expected 570 bp band, while the *Apa*I reaction produced the expected 317 bp band. Thus, the PCR reactions were successful.

Both fragments were excised from the gel, purified, then digested with *Eag*1. The two fragments were then ligated at the *Eag*I site (see page 4). Agarose gel analysis of the ligation reaction shows a fragment of the expected size, demonstrating that the ligation reaction was successful.

The ligated PCR fragment was digested with the restriction enzymes *Apa*I and *Nco*I (page 5) and inserted into the pUC19-91A3H plasmid (pUC19 plasmid containing the gene encoding the variable region of the immunoglobulin heavy chain) at the same sites. The ligated DNA was transformed to *E. coli*, and an *Eag*I digestion of miniprep plasmid preparations (see page 6) was used to identify plasmids having the correct structure.

The above chimeric fragment contained the variable region but not the constant region of the Ig heavy chain. To link this segment with the corresponding constant region of the immunoglobulin gene, the plasmid preparation containing the correct 5.5 Kb fragment was further digested with *Eco*RI, excised from an agarose gel (see page 7), and ligated into a plasmid containing the constant region of the immunoglobulin gene. Page 8 shows an agarose gel of

several *E. coli* minipreps to determine which colonies contain the inserted 5.5 Kb chimeric segment. A digestion analysis of colonies that appeared to contain an insert of the correct size was then performed (see page 9). Colonies were digested with *Eag*I to confirm the insertion of the DNA encoding the antagonist peptide, while *Hind*III was used to confirm colonies with the correct orientation of the inserted DNA. Thus, page 9 of Exhibit A demonstrates the construction of a plasmid encoding an immunoglobulin linked to a T cell receptor antagonist.

Page 10 of Exhibit A shows the cotransfection of the plasmid encoding the above-mentioned chimeric heavy chain with a plasmid encoding a parental light chain of the immunoglobulin to SP2/0 myeloma cells as explained in Example 2 of the specification. Cells that expressed an immunoglobulin heavy chain containing the T cell receptor antagonist (IgPLP-LR) in association with an immunoglobulin light chain were identified by a sandwich radioimmunoassay as described on page 11. The cpm printout of this experiment is shown on page 12 of Exhibit A. Page 13 of Exhibit A shows the screening of specific clones # 7 and # 21 to ensure that they contain both the heavy and light chains of the antibody. Page 14 documents the preparation of the cells expressing the chimeric immunoglobulins for long-term freezer storage.

Page 15 of Exhibit A documents the experiment described in Example 6 of the specification. Briefly, mouse splenocytes were harvested and used as antigen presenting cells for this experiment. The APCs were incubated with the chimeric antibody for 1.5 hours, then mixed with PLP-1 specific T cell hybridomas, and incubated overnight. T cell activation was then measured by IL-2 production, which in turn, was measured by proliferation of IL-2 dependent HT-2 cells as described in the specification. A graph of the results is shown on page 16 of Exhibit A, demonstrating that activation of PLP1-specific T cells by APCs is prevented when the APCs have been previously incubated with Ig-PLP-LR.

The same result can be found in the experiment diagrammed on page 17 (as well as the corresponding data table, page 18, and graph, page 19) of Exhibit A, showing that T cell activation is prevented when APCs have been previously incubated with IgPLP-LR.

The next two experiments clearly demonstrate antagonism of T cell activation by IgPLP-LR. The experiment shown on page 20 (with the corresponding graph on page 21) demonstrates *in vitro* prevention of agonist-mediated T cell activation by IgPLP-LR. The experiment is described in Example 8 (Figure 5A, 5B, and 5C) in the specification. Briefly, APCs were incubated with an agonist (PLP1, IgPLP1, or PLP) with various concentrations of immunoglobulins linked to the antagonist for one hour. PLP1-specific T cells were then added, and T cell activation was measured after an overnight incubation. The results of this experiment demonstrate that IgPLP-LR, when incubated with APCs and a PLP agonist, prevents subsequent activation of PLP1-specific T cells *in vitro*.

The experiment outlined on pages 22 – 23 of Exhibit A is also shown in Example 11 and Figure 8 of the specification. Briefly, mice co-immunized with both IgPLP-LR and IgPLP1 did not experience activation of lymph node (A) or spleen cell (B) T cells upon exposure to the agonist PLP1, while mice injected with IgPLP1 alone produced a strong T cell activation response to subsequent PLP1 administration. Therefore, this experiment demonstrates the *in vivo* prevention of T cell activation.

3. Each of the dates deleted in the 23 pages of the laboratory notebook of Exhibit A is prior to November, 1996.

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4. Thus, Exhibit A demonstrates that the invention was reduced to practice prior to November, 1996 by the preparation and use of a composition comprising a chimeric immunoglobulin containing a T cell receptor antagonist peptide. The chimeric immunoglobulin containing the T cell receptor antagonist was capable of Fc receptor binding, processing, and presentation in association with endogenous MHC Class II molecules to the T cells, thereby preventing T cell activation.

5. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or patent issuing therefrom.

Habib Zaghouani

Date: 08/21/01

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